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AN IMPROVED DYED AMYLOSE FOR PLANT α-AMYLASE ASSAY

INTRODUCTION

CHROMOGENIC SUBSTRATES have been used extensively for α-amylase assays in medical research (Klein et al., 1970; Thoma et al., 1971), but have received only limited attention in agricultural areas (Walter and Purcell, 1973; Bärwald et al., 1970). Since the procedures for determination of α-amylase (α, 1→4-glucan 4-glucanohydrolase, EC 3.2.1.1) in plants (AOAC, 1970; Ikemiya and Deobald, 1966) are time consuming, improved chromogenic substrates could be advantageous for plant α-amylase assays, e.g., in sweet potato flake production where processing conditions are altered relative to the α-amylase activity of the roots (Hoover, 1967; Deobald et al., 1969). Because chromogenic substrates have been reported to be nonreactive with β-amylase (Thoma et al., 1971), and the assay procedures are simple and rapid, effort was directed towards synthesis of a dyed amylose which would be a sensitive substrate for determination of plant α-amylase.

MATERIALS & METHODS

AMYLOSE, Type 1, from potato was purchased from Sigma Chemical Company, and Amylochrome from Scientific Products. For analysis of tobacco α-amylase, the Amylochrome was washed with water and air dried to remove added buffer. South Carolina tobacco (1971), which had been flue-cured and redried, was freeze dried prior to the amylase extract preparation. Tobacco enzyme was extracted in phosphate buffer (0.04M, pH 6.2, containing 0.001M $CaCl_2$) in the presence of Polyclar AT (Loomis and Battaile, 1966) by the procedure of Perez et al., (1971). Sweet potatoes were obtained from the fresh market. A mixture of sweet potato juice plus Celite (about 1.5g/30 ml) was centrifuged, and the extract diluted with buffer.

Synthesis of Amylose-Cibachron Blue F3GA

The initial steps in the substrate preparation were based on the procedure of Klein et al. (1969), with modifications in the amount of Na_2SO_4 (370g/125g amylose) and reaction temperature (60°C). The washed dyed amylose was incubated in water at 50°C for 3-3/4 hr, centrifuged, and the supernatant decanted. The product was incubated at 44°C for 1-1/2 hr in 0.04M phosphate buffer (pH 6.2) containing 0.001M $CaCl_2$, centrifuged, washed with water until the washings were practically colorless, and freeze dried. Three active preparations of the substrate, which is stable at room temperature, were made.

Alpha-amylase assays

New substrate method. Substrate (70 mg) was incubated with 0.2 ml sweet potato extract in 2.3 ml of 0.04M phosphate buffer (pH 6.0) containing 0.001M $CaCl_2$ at 40 ± 0.2°C, as described by Klein et al. (1970). The reaction was terminated with 4.5 ml of 1.8% TCA. Celite (about 0.1g) was added to the supernatant prior to centrifugation. Reaction of 20 mg substrate with enzyme in 1.0 ml incubation volume and 4.2 ml final volume was linear up to an absorbance of 0.35 (625 nm); hence, 20 mg substrate could be used with dilute enzyme extracts. The color of the supernatant after removal from substrate remains stable indefinitely.

Tobacco enzyme was incubated with substrate in phosphate buffer (pH 6.25) containing 0.001M $CaCl_2$ at 37°C; and 0.1M acetate buffer (pH 3.5) was effective in terminating the reaction. Human saliva in 0.9% NaCl was reacted with substrate at 37°C in phosphate buffer (pH 7.0) containing 0.02M NaCl, and the reaction terminated with the acetate buffer. Sample absorbance readings were converted to dye units by reference to a calibration curve constructed as described by Ewen (1973). One unit of enzyme activity is defined as the amount of enzyme which results in liberation of 0.01 micromole of dye per minute under the conditions defined for assay.

Amylochrome procedure. Tobacco α-amylase was determined using water washed Amylochrome following the procedure described for the reaction of the new dyed amylose with tobacco enzyme, except that a 2.0 ml incubation volume was used. The total final volume was 7.0 ml. In the sweet potato enzyme assay, an Amylochrome tablet was dispersed in 2.0 ml of 0.001M $CaCl_2$ at 40°C, the enzyme added, incubated, and the reaction terminated with 2.2 ml of 2.6% or 5.0 ml of 1.8% TCA. Salivary amylase plus water to make 2.0 ml was incubated with an Amylochrome tablet at 37°C, and the enzyme activity terminated with 2.2 ml of acetate buffer, pH 3.5.

AOAC procedure. The AOAC (1970) method for α-amylase modified for use with sweet potatoes (Ikemiya and Deobald, 1966) was followed, except that a dextrin-iodine solution as described by Hasling et al. (1973) was substituted for the comparator.

RESULTS & DISCUSSION

DUPLICATES agreed to ±0.004 absorbance units using 20 mg substrate and ±0.008 absorbance units with 70 mg substrate. Dye units (DU) calculated for a sweet potato sample assayed using 70 mg substrate (0.234 DU/μl) and 20 mg substrate (0.247 DU/μl) were similar. Reaction rates were linear for at least 20 min.

As is evident in Figure 1, good correlation was observed by comparison of results obtained for sweet potato samples assayed using the AOAC (1970) method (Ikemiya and Deobald, 1966) and the new dyed amylose procedure. β-Amylase from sweet potato (Sigma) and β-amylase (Wallerstein) used in preparation of β-limit dextrin did not react with the new substrate when incubated at pH 4.8, 37°C for 15 min, which could be expected from the good agreement found between the AOAC α-amylase procedure and the new method.

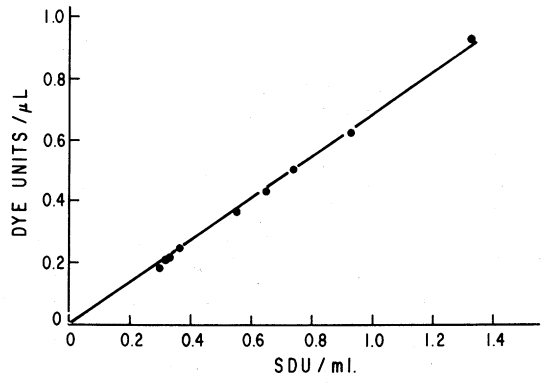

Fig. 1—Comparison of α-amylase activities in sweet potato samples determined by modified AOAC (SDU) and New dyed amylose methods.

The tobacco enzyme released about 45 times as much dye from the improved substrate (Table 1). A manuscript is in preparation on serial tobacco sample analyses. About seven to ten times greater response was obtained with 20 mg of the new substrate than found with Amylochrome tablets (containing 200 mg substrate) in reaction with salivary and sweet potato α-amylases (Table 2). The fact that an absorbance of about 0.03 units was obtained at "zero" enzyme concentration on reaction of sweet potato enzyme with Amylochrome (Fig. 2) in addition to the lower sensitivity observed makes the new substrate more attractive than the commercially available dyed amylose for determination of plant α-amylase.

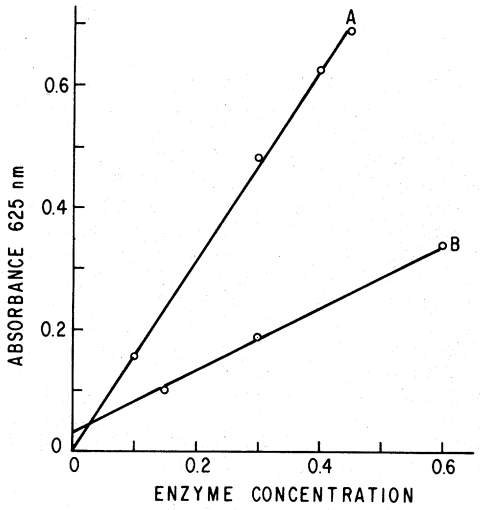

Fig. 2—Reaction of sweet potato α-amylase with increasing enzyme concentration using new substrate (A) and Amylochrome tablets (B).

Table 1—Flue-cured tobacco α-amylase activity observed with Amylochrome[a] and with the new substrate

Substrate	mg Substrate	ml E[b]	Incubation time (min)	Absorbance (625 nm)	Units ml E 15 min
Amylochrome	200	0.20	30	0.065	1
Amylochrome	200	0.40	30	0.113	0.8
Amylochrome	50	0.20	30	0.008	0.1
New dyed amylose	50	0.10	15	0.738[c]	44.7

[a] The Amylochrome was washed with water and air dried prior to use.
[b] Enzyme extract
[c] The amount of dyed amylase used was not sufficient for maximum activity at this level.

Initial studies were made using substrate prepared as described by Klein et al. (1969) for Amylochrome, which gave the same results with tobacco enzyme as those obtained with commercially available Amylochrome. Work on development of the improved substrate included use of starch or amylose from several sources, variations in temperature (50–60°C), and incubation in water or in buffer containing $CaCl_2$. The substantial improvement in substrate was obtained only after treatment with buffer containing $CaCl_2$. Possibly incorporation of calcium into the substrate induced a change in conformation of the substrate which provided energetically favorable binding at the enzyme active sites.

Recently, Walter and Purcell (1973) used the chromogenic substrate Amylopectin Azure (Calbiochem) for analysis of sweet potato α-amylase. However, at 40°C incubation, extrapolation to "zero" enzyme concentration gave an absorbance of about 0.03 units (595 nm), and the linear portion at 40°C extended only to 0.225 absorbance units. At 60°C linearity was observed only up to 0.45 (595 nm). Sweet potato α-amylase shows linearity with the new dyed amylose at least to an absorbance of 0.70 (625 nm). The new substrate procedure was about 3.5 times more sensitive than the Amylopectin Azure method described by Walter and Purcell (1973) for sweet potato enzyme. Since good correlation with the established α-amylase procedure (Ikemiya and Deobald, 1966) was obtained using the dyed amylose (Fig. 1), much time and effort could be saved by using the chromogenic substrate in assaying α-amylase for sweet potato flake production.

The stability of the dyed amylose is a decided advantage over the substrate used in the AOAC procedure. Because of the possible presence of color or inhibitors in plant extracts, the dilute enzyme samples which can be measured provides a particular advantage. The substrate, which is effective with tobacco, sweet potato, and human salivary α-amylases, may have application for enzymes from other sources, e.g., cereal α-amylases. This precise, simple, rapid method offers advantages over established procedures for determination of plant α-amylase.

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Table 2—Yam, sweet potato and human salivary α-amylase activity obtained with Amylochrome tablets and with the new substrate

	Amylochrome (200 mg substrate)				New substrate (20 mg)			
Sample	ml E[b]	E dil.	Absorbance (625 nm)	Units 1.0 ml juice or saliva	ml E[b]	E dil.	Absorbance (625 nm)	Units 1.0 ml juice or saliva
1. Yam	0.30	1→100	0.024[a]	30.0	0.10	1→100	0.118	461
2. Yam	0.40	1→100	0.063	56.0	0.10	1→100	0.170	671
3. Sweet potato	0.10	1→25	0.116	104.5	0.10	1→250	0.101	995
4. Human saliva	0.20	1→20,000	0.070	25,000	0.10	1→20,000	0.244	170,000
5. Human saliva	0.20	1→20,000	0.063	22,400	0.10	1→20,000	0.211	147,000

[a] Since on reaction of sweet potato enzyme with Amylochrome, extrapolation to "zero" enzyme concentration gave an absorbance of about 0.03 units, a correction factor (0.03) was used for the sweet potato and yam results.
[b] Enzyme extract

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